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Genomic Organization of the Rat Pituitary Adenylate Cyclase-activating Polypeptide Receptor Gene

ALTERNATIVE SPLICING WITHIN THE 5′-UNTRANSLATED REGION*

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Tapan K. Chatterjee, Xuebo Liu, Robin L. Davisson‡, and Rory A. Fisher§

From the Departments of Pharmacology and ‡Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

Pituitary adenylate cyclase-activating polypeptide (PACAP) elicits its diverse biological actions by interacting with both PACAP-selective type I PACAP receptors (PACAPRs) and type II PACAPRs that do not distinguish between PACAP and vasoactive intestinal polypeptide. Using long distance polymerase chain reaction, we amplified and characterized the entire coding region of the rat type I PACAPR (rPACAPR) gene, which spans 40 kilobases and contains 15 exons. Mapping of the exons and sequencing of all intron-exon boundaries revealed a structural organization of the rPACAPR gene that is very similar to those encoding other members of the calcitonin/secretin/parathyroid hormone receptor family. Southern blot analysis demonstrated a single copy of the rPACAPR gene. A combination of rapid amplification of cDNA ends and reverse transcriptase polymerase chain reaction revealed an unexpected diversity in the rPACAPR mRNA in the 5′-untranslated (5′-UTR) region. Four rPACAPR cDNAs were identified with 5′-UTR sequences that all diverged from the genomic sequence at a site 76 bp upstream of the ATG start codon, where a consensus 3′ slice acceptor sequence was located. Sequence analysis of these amplified transcripts demonstrated that they arise by tissue-specific differential usage of four exons in the 5′ noncoding region of the rPACAPR gene. This study is the first to elucidate the structural organization of a PACAPR gene and to demonstrate that alternative splicing generates rPACAPR transcripts with unique 5′-UTRs.

In 1989, Arimura and colleagues (1) discovered a novel bioactive peptide in their attempt to identify new hypothalamic hormones regulating anterior pituitary hormone secretion. This peptide was named pituitary adenylate cyclase-activating polypeptide (PACAP),[1] reflecting its potent ability to stimulate increases in cAMP in cultured rat anterior pituitary cells. Structural studies revealed the peptide to be a C-terminal amidated 38-amino acid peptide (PACAP-38), and subsequent studies resulted in identification of the other molecular form of PACAP (PACAP-27) (2), formed by proteolytic processing of the encoded PACAP precursor protein. PACAP is a member of the VIP/secretin/glucagon/GRF family of neuropeptides. Immunochemical studies have revealed the existence of PACAP-containing nerve fibers throughout the central and peripheral nervous system, and radioimmunoassay results have demonstrated a broad distribution and range of tissue concentrations of the two molecular forms of PACAP (3–5).

PACAP possesses an impressive array of biological actions consistent with its diverse tissue distribution and suggested roles as hypophysiotropic hormone, neurotransmitter, neuromodulator and vasoregulator (5, 6). PACAP stimulates hormone release from various cells (1, 7–13) and is the most potent insulin secretagogue yet described (14). PACAP stimulates neurite outgrowth in pheochromocytoma PC-12 cells (15), promotes mitogenesis and survival of cultured rat sympathetic neuroblasts (16, 17), and prevents neuronal cell death induced by human immunodeficiency virus protein gp120 in dissociated hippocampal cultures (5). PACAP dilates various vessels (5, 18), induces hypotension (1, 19), stimulates steroidogenesis (20), and stimulates hepatic glycogenolysis (21). A multifunctional role of PACAP is suggested further by the presence of dense networks of PACAP fibers and/or high affinity binding sites for PACAP in these target tissues (reviewed in Ref. 5).

PACAP produces its biological effects by interacting with at least two types of high affinity receptors, type I PACAP-preferring receptors and type II receptors, which do not distinguish between PACAP and VIP (5, 22, 23). Recombinant type II receptors (24, 25) bind VIP and PACAP with equal affinity and activate adenylyl cyclase. Based upon their pharmacological properties and tissue distribution, these receptors appear to represent the known VIP receptor (*i.e.* the receptor that has shared ligand specificity for PACAP). The type I PACAPR, cloned by several laboratories (26–31), binds PACAP with an affinity approximately 1000 times higher than VIP and activates adenylyl cyclase. Spengler *et al.* (30) described five splice variants of this receptor differing in the region corresponding to the third intracellular loop of the receptor. Four of these five splice variants exhibited the multifunctional signaling capability of PACAP receptors described in several cell types (15, 32, 33), activating both adenylyl cyclase and phosphoinositide phospholipase C. Structurally, the PACAPR exhibits sequence

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U82669, U84740, U84741, U84742, and U84743.

§ To whom correspondence should be addressed: Department of Pharmacology, University of Iowa, Iowa City, IA 52242. Tel.: 319-335-8331; Fax: 319-335-8930.

[1] The abbreviations used are: PACAP, pituitary adenylate cyclase activating polypeptide; PACAPR, PACAP receptor; rPACAPR, rat PACAPR, VIP, vasoactive intestinal polypeptide; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; RACE, rapid amplification of cDNA ends; kb, kilobase(s); bp, base pair(s); UTR, untranslated region; G protein, guanine nucleotide-binding protein; GRF, growth hormone-releasing factor; mGRFR, mouse GRF receptor; PTH, parathyroid hormone; mPTHR, mouse PTH receptor; rCRFR, rat corticotropin-releasing factor receptor; pCTR, porcine calcitonin receptor.

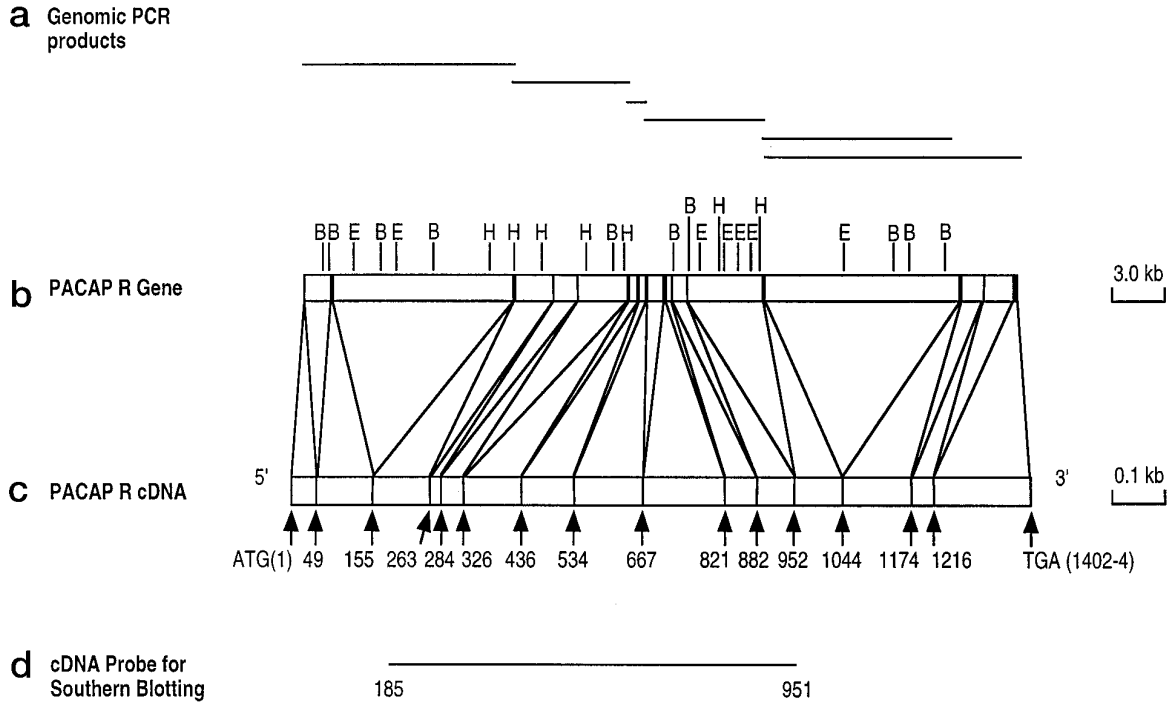

FIG. 1. **Organization of the rPACAPR gene in relation to its mRNA coding sequence.** *a*, positions of the genomic PCR products used to map the gene. *b*, restriction map and intron-exon organization of the gene. Positions of exons (*filled*) and introns (*open*) are shown aligned with the common restriction sites. *c*, structure of the PACAPR cDNA. The locations of the introns are indicated by the nucleotide number on the cDNA, with base 1 corresponding to the "A" of the ATG start codon. *d*, cDNA probe used for Southern blotting (nucleotides 185–951 of rPACAPR cDNA).

homology to the new family of G protein-coupled receptors first identified by cloning of secretin, calcitonin, and PTH receptors (34–36).

The understanding of the mechanisms involved in PACAPR expression at transcriptional and translational levels and the physiologic role of the PACAPR gene would be facilitated greatly by elucidation of the structure and processing of its gene. In this report, we describe the complete structural organization of the coding region of the rPACAPR gene. We demonstrate that this gene consists of at least 15 exons and 14 introns with an intron-exon structure highly conserved among other known genes in the secretin/calcitonin/PTH family of G protein-coupled receptors. We also provide the first evidence for tissue-specific differential splicing in the 5′-UTR of the rPACAPR mRNA generating rPACAPR transcripts with unexpected diversity in the 5′-UTR.

MATERIALS AND METHODS

PCR Amplification of Genomic DNA Fragments of the rPACAPR Gene—Overlapping fragments of genomic DNA encompassing the entire coding sequence of the rPACAPR gene (Fig. 1) were amplified from rat genomic DNA (CLONTECH) by long distance PCR using Elongase (Life Technologies, Inc.) and a series of forward and reverse primers to rPACAPR cDNA sequences. The routine PCR mixture (50 μl) contained 60 mM Tris-SO_4, pH 9.1, 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.2 μM primers, and 2 units of Elongase enzyme mix. Amplification was performed for 35–40 cycles with denaturation at 94 °C for 30 s followed by annealing/extension at 68 °C for 5–15 min. Automated fluorescent dideoxy sequencing of the purified PCR products was performed at the University of Iowa DNA Core Facility.

PCR Amplification of the ATG 5′-Flanking Sequence of the rPACAPR Gene—The ATG 5′-flanking sequence of the rPACAPR gene was PCR-amplified using the Rat Promoter Finder kit (CLONTECH). Briefly, the supplied rat genomic DNA, digested with various restriction enzymes and ligated at both 5′ and 3′ ends with an adaptor DNA of known sequence, served as templates in PCR using a forward primer to the adaptor sequence and a reverse primer to sequences unique to the rPACAPR cDNA. The 5′-flanking sequence was obtained by automated fluorescent dideoxy sequencing of the purified PCR products (University of Iowa DNA Core Facility).

PCR Determination of Intron Sizes and Restriction Enzyme Mapping of the rPACAPR Gene—The sizes and locations of introns within genomic DNA PCR products were determined by a combination of agarose gel electrophoresis and DNA sequencing. By comparison of the sequence of the amplified genomic DNA to that of the rPACAPR cDNA we were able to confirm the rPACAPR cDNA sequence and to identify intron-exon boundaries and thereby also determine the sizes of the introns and exons.

Restriction mapping of the rPACAPR gene was performed by digestion of the overlapping genomic DNA PCR products with *Eco*RI, *Bam*HI, *Bgl*II, *Hin*dIII, and *Sac*I followed by agarose gel electrophoresis.

Genomic Southern Blot Analysis—Rat genomic DNA (15 μg) was digested with restriction enzymes *Eco*RI, *Bam*HI, *Bgl*II, *Hin*dIII, and *Sac*I, electrophoresed on a 0.8% agarose gel, and blotted to nitrocellulose membrane. The membrane was hybridized with a 767-bp cDNA probe (nucleotides 185–951 of the rPACAPR cDNA) (Fig. 1) labeled with [α-^{32}P]CTP (800 Ci/mmol, Amersham Corp.) by the random priming method. Hybridization was performed overnight at 65 °C in 1 × SSC, 5 × Denhardt's solution, 0.1% SDS, 50 μg/ml salmon sperm DNA, 10% dextran sulfate and then washed with 0.1 × SSC, 0.1% SDS at 65 °C. Autoradiography was performed for 4 days at −70 °C.

Isolation by Anchored PCR of the 5′ cDNA Ends of the rPACAPR—Oligo(dT)-primed cDNA from rat spinal cord was ligated to a synthetic oligonucleotide of known sequence at its 3′ end essentially as we described (37). This anchor-ligated cDNA served as the template for PCR amplification of its 3′ end (*i.e.* corresponding to the 5′ mRNA end) using a forward primer complementary to the anchor sequence and a reverse primer specific to sequences of the rPACAPR cDNA. Two rounds of seminested PCR (*i.e.* with different reverse primers and the same forward primer) resulted in amplification of products ranging in size from approximately 250 to 900 bp. The reverse primer used in the final round of PCR corresponded to nucleotides 783–807 of the rPACAPR cDNA, suggesting that the largest PCR product extended approximately 100 bp into the 5′-UTR region of the rPACAPR cDNA. PCR products were cloned, and multiple colonies were selected for sequencing.

RT-PCR Analysis of Tissue-specific Differential Splicing of 5′-UTR of rPACAPR mRNA—Oligo(dT)-primed cDNA from various rat tissues or cells (pancreatic β-islets) were amplified in two successive rounds of seminested PCR using a forward primer corresponding to a sequence found within the 5′-UTR (GTCTGGACCGGCCCGGAGACCAG, ob-

TABLE I

Intron sizes and splice junction sequences in the coding region of the rPACAPR gene

Exon and intron sequences are represented by capital and lowercase letters, respectively. Base pairs in exons are grouped in codons.

Exon-intron junction sequence		Intron number	Intron size
5′ donor site	3′ acceptor site		
			kb
CTG CTG CCT GTG gtaagg.....cctacagGCT ATT GCT ATG CAC		1	1.5
Leu Leu Pro Val	Ala Ile Ala Met His		
GAG TCT TCC CCA Ggtgagtg.....ctctcctccagGT TGC CCT GGC ATG		2	10.2
Glu Ser Ser Pro G	ly Cys Pro Gly Met		
AAC CCG GAC CAA Ggtagg.....acatacagTC TGG ATG ACA GAA		3	2.2
Asn Pro Asp Gln V	al Trp Met Thr Glu		
ACC ATA Ggtaagagg.....tacagGA GAT TCT GGT		4	1.3
Thr Ile G	ly Asp Ser Gly		
TCC TTG GAG ATC ACA Ggtaagg.....ctcttcagAC ATG GGG GTC		5	2.8
Ser Leu Glu Ile Thr A	sp Met Gly Val		
CCT GAG TCT GGA GAT CAGgtaagt.....tccag GAT TAT TAC TAC CTG		6	0.46
Pro Glu Set Gly Asp Gln	Asp Tyr Tyr Tyr Leu		
ATC TTG TGC CGC TTC CGgtgaga.....ccacgcagG AAG CTG CAT TGC		7	0.37
Ile Leu Ser Arg Phe Ar	g Lys Leu His Cys		
TGC TTC GTT TCC ACCgtaagtg.....ctgtagGTG GAG TGC AAA GCT		8	0.90
Ser Phe Val Ser Thr	Val Glu Cys Lys Ala		
ATC ATC GGC TGG Ggtaggt.....tccagGG ACA CCT ACT GTG TGT		9	0.32
Ile Ile Gly Trp G	ly Thr Pro Thr Val Cys		
TTT GAT GAT GCA GGgtaagt.....ctttcagA TGC TGG GAT ATG AAT		10	0.62
Phe Asp Asp Ala Gl	y Cys Trp Asp Met Asn		
CCC CTG GTT GGC TCT ATA ATGgtgagt...cactcctcagGTT AAC TTT		11	4.2
Pro Val Val Gly Ser Ile Met	Val Asn Phe		
TCC AGC ATC TAC TTgtaagt.....tgacagA CGG CTG GCC CGC TCC		12	10.5
Ser Ser Ile Tyr Le	u Arg Leu Ala Arg Ser		
CTG GGC TCC TTC CAGgtagg.....cccagGGC TTT GTG GTG GCT		13	1.93
Leu Gly Ser Phe Gln	Gly Phe Val Val Ala		
TTC CTC AAT GGG GAGgtaagac.....ttgcagGTA CAG GCA GAG ATT		14	1.57
Phe Leu Asn Gly Glu	Val Gln Ala Glu Ile		

tained by anchored PCR) of the rPACAPR transcript. In the first round of PCR, this forward primer was used in combination with a reverse primer corresponding to nucleotides 292–325 of the rPACAPR cDNA. In the final round of PCR, the same forward primer was used in combination with a nested reverse primer corresponding to nucleotides 230–262 of the rPACAPR cDNA. PCR-amplified products were resolved by agarose gel (2%) electrophoresis, and selected products were extracted from the gel, purified, and subjected to DNA sequencing.

Primer Extension Analysis—Poly(A)$^+$ RNA was isolated from rat cerebellum and rat cerebral cortex using a commercial kit (Invitrogen). Poly(A)$^+$ RNA from rat cerebral cortex (7 μg) and rat cerebellum (5 μg) and yeast tRNA (30 μg) were reverse transcribed with avian myeloblastosis reverse transcriptase using a ^{32}P-end-labeled oligonucleotide (1×10^5 cpm) corresponding to nucleotides -1 to -37 of the rPACAPR cDNA. Reaction products were separated on a denaturing 9% polyacrylamide gel using the sequence of the rPACAPR cDNA as a size standard.

RESULTS

Structure of the rPACAPR Gene—To determine the structure of the coding sequence of the rPACAPR gene, we performed long distance PCR with rat genomic DNA and primers to rPACAPR cDNA sequences to systematically walk up the entire coding sequence of the rPACAPR gene. Amplification by *Taq* polymerase is limited generally to DNA templates less than 5 kb because of its inability to correct misincorporations via 3′ to 5′ exonuclease activity. To accomplish amplification of long segments of genomic DNA and to assure the fidelity of amplification, we used a commercially available mixture (Elongase) of *Taq* polymerase and *Pyrococcus* sp. GB-D DNA polymerase, the latter exhibiting 3′ to 5′ exonuclease activity. Using this strategy, we successfully amplified six overlapping genomic DNA segments ranging in size from 1.1 to 11.9 kb that spanned the entire coding sequence of the rPACAPR gene (Fig. 1). By sequencing these PCR products, we were able to confirm the rPACAPR cDNA sequence and identify the locations and sizes of introns. Alignment of these genomic PCR products demonstrated that the coding region of the rPACAPR gene spans 40 kb of DNA and is interrupted by 14 introns. The intron-exon organization of the rPACAPR gene in relation to its mRNA is shown in Fig. 1.

Table I shows the sizes of introns and the intron-exon splice junction sequences in the coding region of the rPACAPR gene. As shown, the introns vary in size from 320 bp to 10.5 kb, and all of the splice acceptor and donor sequences agree with the GT/AG consensus sequence (38). The rPACAPR gene intron splice phasing is type 0 (the intron occurs between codons) for introns 1, 6, 8, 11, 13, and 14; type 1 (the intron interrupts the first and second base of the codon) for introns 2, 3, 4, 5, and 9; and type 2 (the intron interrupts the second and third base of the codon) for introns 7, 10, and 12.

The relationship between exon and intron locations to proposed structural domains of the rPACAPR is shown in Fig. 2. Exons range in size from 21 bp (exon 4) to more than 185 bp (exon 15). The N-terminal extracellular domain of the receptor is encoded by the first six exons and part of the seventh exon. Transmembrane domains I, II, III, and VI are entirely within exons 7, 8, 9, and 13, respectively, while transmembrane domains IV, V, and VII are each interrupted by a single intron. Intracellular domains 1, 2, and 4 are either intronless (2i) or have a single intron located one amino acid from the intracellular domain/transmembrane domain junction (1i and 4i). However, the third intracellular domain (3i) is interrupted by an intron located four amino acids from transmembrane VI. This intron (intron 12) is the largest intron present in the coding sequence of the rPACAPR and is located precisely where alternative splicing can occur to produce the third intracellular loop splice variant forms of the PACAPR (30). Indeed, we

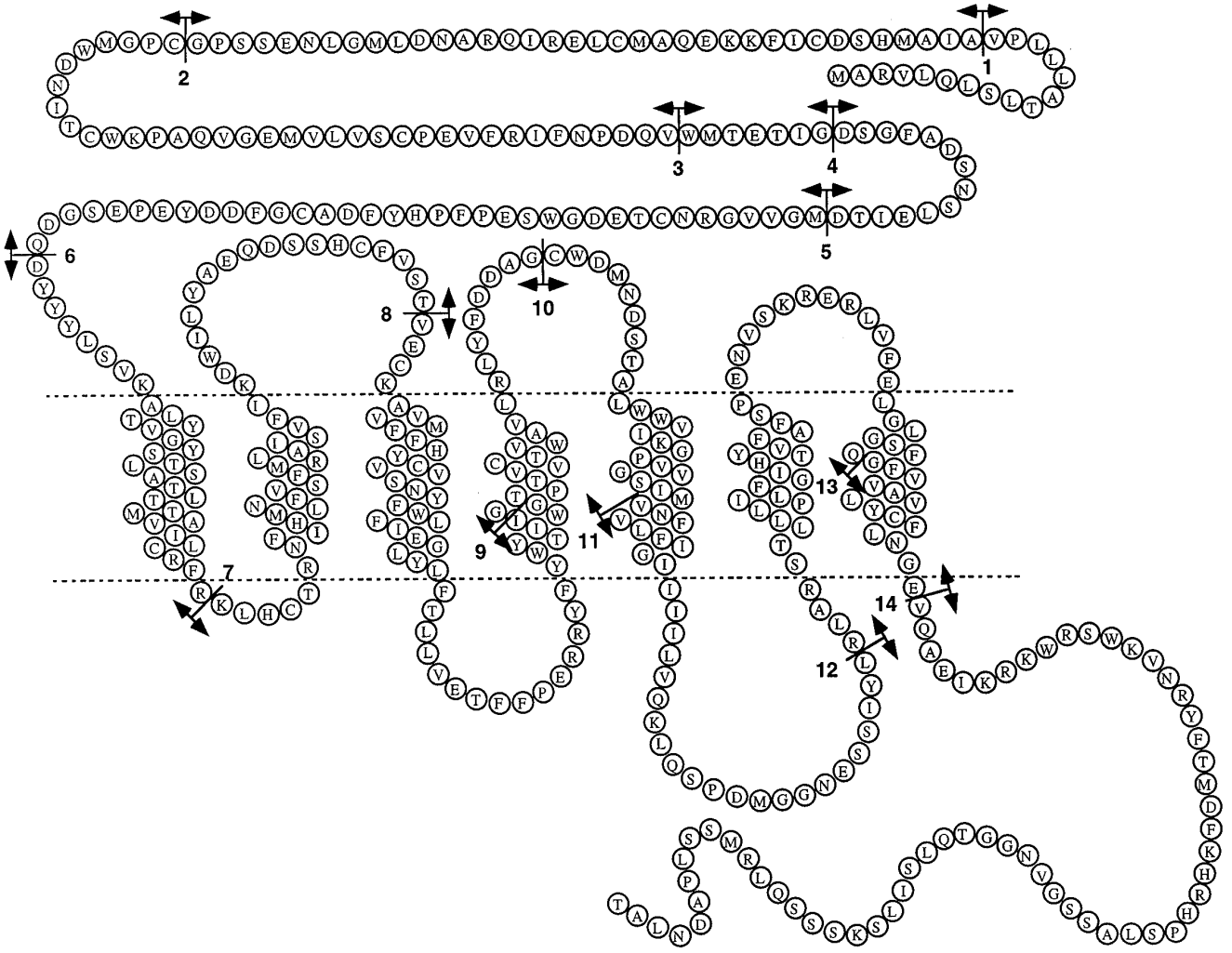

FIG. 2. **Putative membrane topography of the rPACAPR showing the positions of introns.** Locations of introns are indicated by *arrows* and *numbers* that correspond to the coding sequence introns described in Table I.

identified alternate 3′ splice acceptor sites located 2.8 and 7.7 kb from the 5′ end of intron 12 that represent the splicing sites used to produce the hip and hop cassette forms of the rPACAPR, respectively, described by these workers. Introns interrupt two of the three extracellular loops of the rPACAPR. Thus, the regions of the rPACAPR gene coding for the intracellular domains of the receptor are largely intronless compared with the regions coding for the extracellular receptor domains. Also, four of the seven transmembrane domains of the rPACAPR are encoded by individual exons.

Genomic Southern Blot Analysis of the rPACAPR Gene—Southern blot analysis of rat genomic DNA digested by *Eco*RI, *Bam*HI, *Bgl*II, *Hin*dIII, or *Sac*I was performed using a cDNA probe covering exons 3–11 of the rPACAPR gene (nucleotides 185–951 of the rPACAPR cDNA) (Fig. 1). This region of the rPACAPR gene was mapped entirely with each of these restriction enzymes, although Fig. 1 shows only the *Eco*RI, *Bam*HI, and *Hin*dIII sites for purposes of clarity. Fig. 3 shows the results of the Southern blot, and Table II shows the predicted and actual sizes of the fragments recognized by the cDNA probe. As shown, the observed patterns of hybridization were consistent with the restriction sites we mapped in the rPACAPR gene coding sequence. These results confirm the structural organization of the rPACAPR gene. They indicate also that the rPACAPR is encoded by a single gene and that no pseudogenes are present in the rat genome.

Analysis of the ATG 5′-Flanking Region of the rPACAPR Gene—Nearly 1.2 kb of the ATG 5′-flanking sequence (GenBank™ accession number U82669) of the rPACAPR gene was PCR amplified from restriction enzyme-digested, anchor-ligated genomic DNA. Comparison of 5′-UTR sequences of rPACAPR cDNAs to this ATG 5′-flanking sequence of the rPACAPR gene revealed that the region upstream of nucleotide −76 represents part of an intron (4 kb) that is located between the 3′ splice acceptor site and exon 2 of the 5′-UTR of the rPACAPR gene (see below).

5′-UTR Splice Variants of the rPACAPR—To compare the 5′-flanking sequence of the rPACAPR gene to the 5′-UTR of the rPACAPR cDNA, we performed 5′-RACE with rat spinal cord cDNA to amplify the 5′ ends of the rPACAPR cDNA. Using this approach, we amplified and cloned two cDNAs with 5′-UTR sequences beginning −81 and −102 nucleotides from the translation start site, which we named cDNA 1 and cDNA 2, respectively. As shown in Fig. 4, cDNA 2 was identical to cDNA 1 in its 5′-UTR sequence up to nucleotide −79, where it then diverged from cDNA 1 over the next two bases and possessed an additional 21 bp of 5′ sequence. Interestingly, both cDNA 1 and cDNA 2 are identical to the genomic DNA sequence up to nucleotide −79, whereupon they both diverge. In view of these findings, it is particularly noteworthy that there is a consensus splice acceptor sequence located at nucleotide −77 in the 5′-flanking sequence of the rPACAPR gene. The observed divergence in sequence of cDNA 1 and cDNA 2 from each other and from the genomic DNA sequence as well as the existence of a

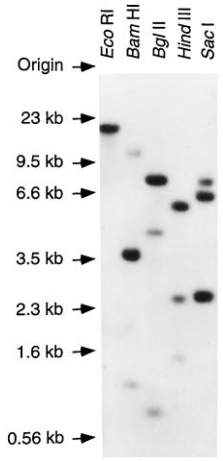

FIG. 3. **Southern blot analysis of the PACAPR gene.** Samples of rat genomic DNA (15 μg) digested with restriction endonucleases *Eco*RI, *Bam*HI, *Bgl*II, *Hind*III, and *Sac*I were electrophoresed, blotted, and hybridized with a ^{32}P-labeled PACAPR cDNA probe (see Fig. 1). Molecular weight markers are indicated on the *left*.

splice acceptor site in this region suggested alternative splicing of the rPACAPR gene in the 5′ noncoding region.

We obtained further evidence for alternative splicing of the rPACAPR gene in the 5′ noncoding region by amplifying two additional rPACAPR cDNAs with unique 5′-UTR sequences. Because cDNA 1 diverged from cDNA 2 by only 2 bp at its 5′ end, we considered the possibility that it represented a truncated form of cDNA 2 and that the 2-bp divergence may have resulted from a PCR artifact, 5′ anchor ligation artifact, or cloning artifact, although five individual clones exhibited the identical sequence of cDNA 1. To examine this possibility, we performed PCR using the unique sequence at the 5′ end of cDNA 2 as a forward primer, rather than the anchor primer, with reverse primers to sequences present in the coding region of the rPACAPR cDNA. Seminested PCR was performed using reverse primers to nucleotides 292–325 and 230–262 of the rPACAPR cDNA in the first and second rounds of PCR, respectively, with direct sequencing of the PCR products. We used rat cerebral cortex and rat liver cDNA as templates in these reactions. We gel-purified the major PCR product amplified from cerebral cortex cDNA and the largest PCR product amplified from rat liver cDNA. Unexpectedly, we amplified two additional rPACAPR cDNAs with unique 5′-UTR sequences that we named cDNA 3 (liver) and cDNA 4 (cerebral cortex).

Alignment of cDNAs 1–4, as shown in Fig. 4, reveals the existence of a unique pattern of alternative splicing in the noncoding region of the rPACAPR gene. As shown, cDNA 3 is identical to cDNA 4 except for the presence of an intervening sequence of 94 nucleotides. This intervening sequence begins precisely at the putative splice junction sequence (nucleotide −77) that we identified in the 5′-flanking sequence of the rPACAPR gene. This strongly suggests that this sequence represents a 3′ splice acceptor site. None of the cDNAs match the genomic sequence beyond nucleotide −79, further supporting alternative splicing of the rPACAPR transcript to produce mRNAs with unique 5′-UTRs.

We performed PCR using genomic DNA as template to identify the genomic locations and intron-exon junction sequences encoding the 5′-UTRs of cDNAs 2–4. As described above, we amplified 5 kb of 5′-flanking sequence of the rPACAPR gene. Although we did not sequence this entire segment of 5′-flanking DNA, we sequenced nearly 1.5 kb from each end, and we were unable to find a noncoding exon matching the 23-nucleotide sequence present at the 5′ ends of cDNAs 2–4. We hypothesized that this 23-nucleotide sequence was upstream of this

TABLE II
Comparison of the sizes of restriction enzyme digestion products of the rPACAPR gene determined by Southern analysis with those predicted by restriction mapping of the rPACAPR gene

Actual sizes were determined from the Southern blot shown in Fig. 3.

Treatment	Predicted size	Actual size
	kb	
*Eco*RI	17.1	17.0
*Bam*HI	11.0	11.4
	4.0	3.9
	1.2	1.2
*Bgl*II	7.0	7.9
	4.9	4.8
	0.8	0.8
*Hind*III	5.7	5.6
	2.4	2.6
	2.4	2.6
	1.6	1.5
*Sac*I	7.2	7.5
	6.1	6.5
	2.6	2.6

region, and we performed PCR using the 23-nucleotide sequence as a forward primer with a reverse primer to a sequence 3.5 kb upstream of the translation start site (*i.e.* −3.5 kb). Using this approach, we amplified a single product of 4.5 kb from rat genomic DNA, suggesting that the 23-nucleotide sequence in question is present in the genomic DNA approximately 8 kb upstream of the translation start site. Sequence analysis of this PCR-amplified genomic DNA revealed the presence of a 5′ splice donor sequence (gtaag) at the 3′ end of the conserved 26-bp sequence found in cDNAs 2–4. We used a similar approach to locate the sequence shared by cDNAs 3 and 4, which is contiguous with the 3′ end of this conserved 26-bp sequence. We found that this shared sequence is located approximately 3 kb downstream of the 26-bp sequence in the genomic DNA, and we identified a splice acceptor sequence (atgag) at its 5′ end and a splice donor sequence (gtaag) at its 3′ end. In addition, we identified the unique sequence of cDNA 3 800 bp downstream of the shared sequence in the genomic DNA with a splice acceptor sequence (catag) at its 5′ end and a splice donor sequence (gtaag) at its 3′ end. These findings indicate splicing of intronic sequences to generate cDNAs 2–4.

Therefore, our results suggest that there exist at least four exons (exons 1–4) upstream of the consensus splice acceptor site located 5′ to nucleotide −76. Exon 1 is located approximately 3 kb upstream of exon 2 and 8 kb upstream of the translation start site, while exon 3 is located approximately 800 bp downstream of exon 2. The location of exon 4 in relation to these three exons, however, is not known. Based on these findings, we proposed a scheme (Fig. 5) to account for the generation of rPACAPR transcripts with unique 5′-UTRs. According to this scheme, cDNA 3 is generated by splicing together exons 1, 2, and 3 to the acceptor sequence at −76. Skipping exon 3 produces cDNA 4, while skipping both exon 2 and exon 3 generates cDNA 2. Similarly, cDNA 1 results from splicing exon 4 with the acceptor sequence (also see "Discussion").

Tissue-specific Generation of 5′-UTR Splice Variants of the rPACAPR—Although cDNAs 2–4 have unique 5′-UTRs, they share sequence derived from exon 1 (Fig. 5). This enabled us to examine whether these unique PACAPR transcripts are expressed in a tissue-specific fashion by analysis of products amplified from various tissues by RT-PCR. We performed seminested PCR using a forward primer corresponding to sequences present in exon 1 with nested reverse primers corresponding to coding sequences of the rPACAPR cDNA. This approach enabled us to amplify specifically rPACAPR transcripts retaining exon 1 at their 5′ ends. As shown in Fig. 6, several products

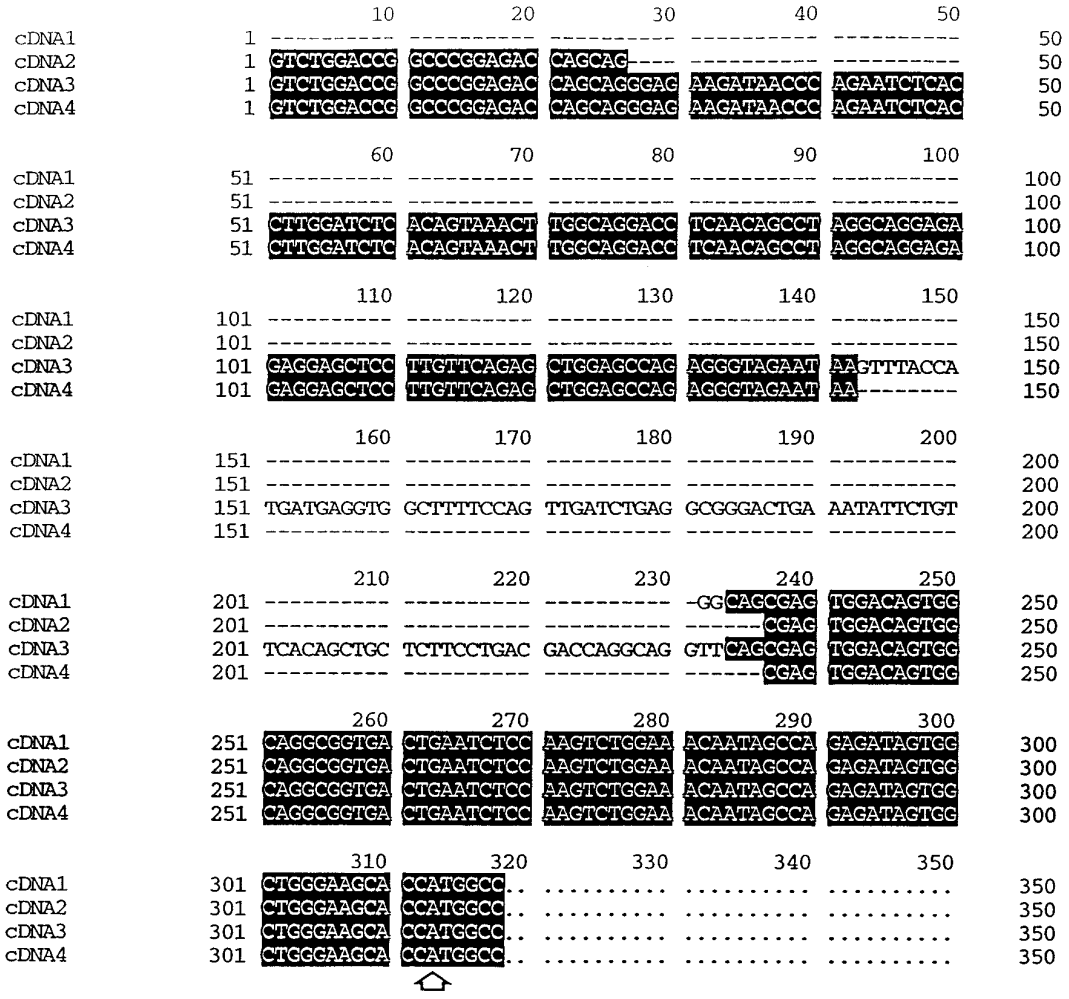

FIG. 4. **Alignment of nucleotide sequences of 5′-UTR of cDNAs 1–4.** The first nucleotide of the ATG start codon is designated with an *arrow*. GenBank™ accession numbers for cDNAs 1, 2, 3, and 4 are U84741, U84740, U84743, and U84742, respectively.

were amplified from most tissues examined. These products ranged in size from approximately 350 to 575 bp, although several larger products were present in kidney and no amplification products were apparent in pancreatic β-islets. The three major products present in most of the tissues examined, although in different relative amounts in different tissues, were gel-purified and sequenced. The sizes of these products corresponded exactly to the predicted products expected for cDNAs 2–4, and sequence analysis of these three products, labeled *574*, *480*, and *360 bp* in Fig. 6, confirmed their identity to cDNAs 3, 4, and 2, respectively. Because the PCR conditions were not biased for amplifying any one of these three cDNAs (*i.e.* each cDNA was amplified with the same combination of primers), the relative amounts of the PCR products corresponding to cDNAs 2–4 would be expected to be proportional to their tissue abundance. Fig. 6 shows clear differences in the PCR products corresponding to these three cDNAs. For instance, the 574-bp product (cDNA 3) appears to be a major transcript in liver and lung but not in brain. Although we have amplified coding sequences of the rPACAPR from pancreatic β-islet cells (not shown), the absence of products corresponding to cDNAs 2–4 in this tissue suggests that exon 1 is not retained in PACAPR transcripts in these cells. Together, these results demonstrate differential splicing of the rPACAPR transcript to produce rPACAPR mRNAs with unique 5′-UTRs.

Fig. 6 also shows amplification of an additional 400-bp product in several tissues and of products larger than 574 bp in kidney. Whether these products represent additional splice variants of the rPACAPR that utilize an exon(s) not present in cDNAs 2–4 remains to be determined.

Identification of the Transcription Start Site—Fig. 7 shows transcription start site mapping by primer extension on rat cerebral cortex and cerebellum poly(A)$^+$ RNA. An antisense primer corresponding to nucleotides −1 to −36 of the sequence present in cDNAs 1–4 (Fig. 4) was used in the primer extension to reveal multiple products ranging from approximately 80 to 107 nucleotides. Because the extension primer we used began at nucleotide −1, the lengths of the extension products correspond to the position of the transcription start site relative to the translation start site. The major extension product was 95–97 nucleotides in length with minor extension products of 105–107 and 82 nucleotides in length. These same bands were present in the cerebellum samples (although not as apparent in this autoradiogram) but were not present in the tRNA sample even upon overexposure of the autoradiogram. The products less than 75 nucleotides in length were nonspecific, since they were observed in the tRNA lane in other experiments.

The lengths of the 5′-UTR sequence of the two cDNAs we amplified by 5′ RACE (*i.e.* cDNA 1, 81 nucleotides; cDNA 2, 102 nucleotides) are in fairly good agreement with the extension products of the 82 and 105–107 nucleotides. Although Fig. 7 shows sequences only up to approximately 190 nucleotides in length, no extension products of up to 310 nucleotides in length were observed. Thus, no extension products were found with lengths comparable with the lengths of the 5′-UTR of cDNA 3 (212 bp) and cDNA 4 (301 bp). In view of these findings and the

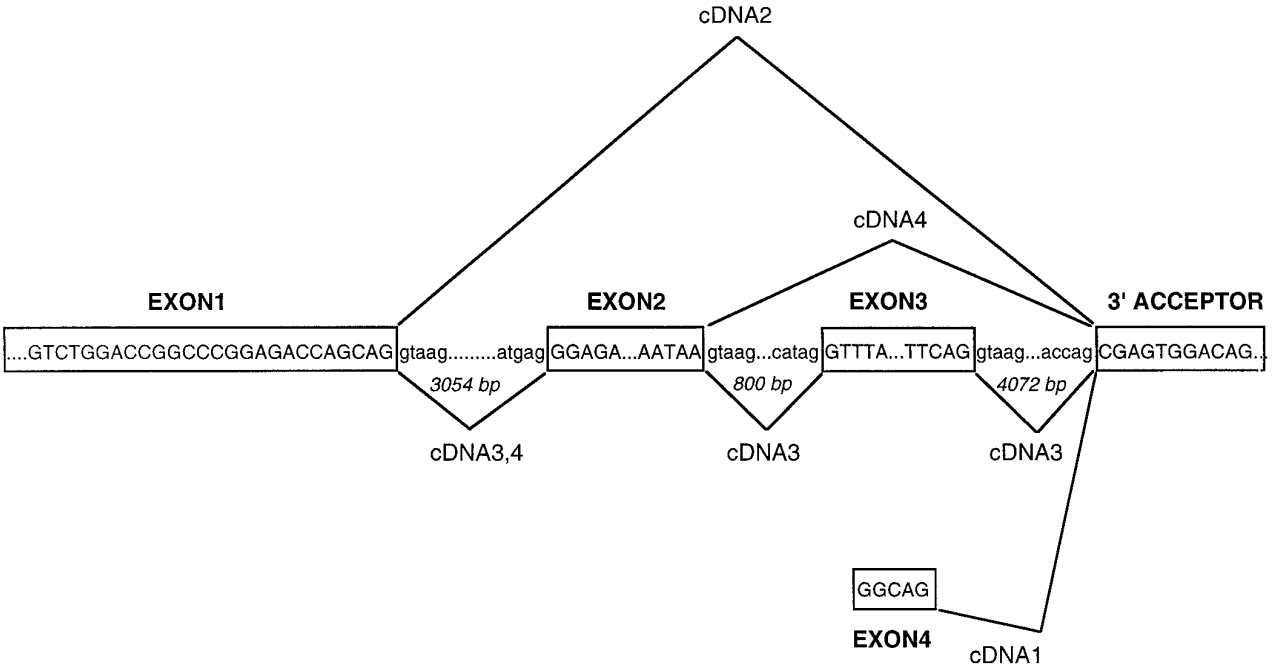

FIG. 5. **Representation of the splicing events occurring in the 5′-UTR of the rPACAPR gene to generate cDNAs 1–4.** Exon sequences are in *capital letters* and *boxed*. Splice donor and acceptor sequences within introns are in *small letters*. Intron sequences were identified in the genomic DNA and were contiguous with the indicated exon sequences. The location of exons 1–3 was determined by PCR, and the distance in bp between exons is indicated in the *figure*.

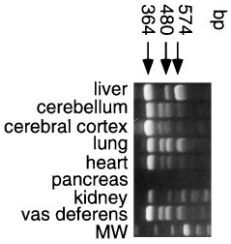

FIG. 6. **Tissue-specific differential splicing of the 5′-UTR of the rPACAPR transcript.** Oligo(dT)-primed cDNAs from various rat tissues that express PACAPRs were subjected to seminested PCR as described under "Materials and Methods." The forward primer used was specific to exon 1 of the 5′-UTR of the rPACAPR mRNA with the nested reverse primers complementary to nucleotides 292–325 (round 1) and 230–262 (round 2). PCR products were resolved by 2% agarose gel electrophoresis. The lane marked *MW* represents a 100-bp DNA ladder. The *arrows* indicate the expected sizes of cDNAs 2–4 (Fig. 5).

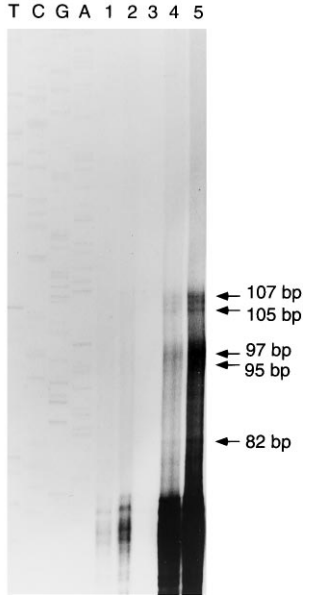

FIG. 7. **Identification of transcription start sites on the rPACAPR gene by primer extension.** Primer extension was performed with rat cerebellum poly(A)$^+$ RNA, rat cerebral cortex poly(A)$^+$ RNA, or yeast tRNA as described under "Materials and Methods." Extended products of these reactions were subjected to denaturing polyacrylamide electrophoresis. *Lanes 1* (2 μg) and *2* (3 μg), rat cerebellum poly(A)$^+$ RNA; *lane 3*, yeast tRNA (9 μg); *lanes 4* (2.8 μg) and *5* (4.2 μg), rat cerebral cortex poly(A)$^+$ RNA. The sizes of the extended products in *lanes 4* and *5* are indicated by *arrows*. The double-stranded sequencing reaction shown on the *left*, used to determine the size of the products described above, was produced by sequencing the rPACAPR cDNA clone.

fact that these two cDNAs were not amplified by 5′ RACE, it is likely that their 5′ sequence is incomplete. Since cDNA 2 shares the same 5′ sequence with cDNAs 3 and 4, it is possible that it too represents an incomplete transcript (see "Discussion").

DISCUSSION

In the present study, we have elucidated the structure of the coding region of the rPACAPR gene, including its ATG 5′-flanking region, and provided evidence for tissue-specific differential splicing of the rPACAPR mRNA in the 5′-UTR. The coding region of the rPACAPR gene is composed of 15 exons and spans 40 kb of genomic DNA. The first exon, encompassing the translation start site, extends into the 5′ noncoding region of the rPACAPR gene and together with exons 2–7 encodes the large N-terminal extracellular domain of the receptor. Exons 7–14 encode the seven transmembrane domains and associated intra- and extracellular loops, and exon 15 encodes the C-terminal cytoplasmic tail of the receptor and extends into the 3′ noncoding region of the rPACAPR gene.

The intron-exon organization of the rPACAPR gene is strikingly similar to that of genes encoding other members of the group III family of G protein-coupled receptors (*i.e.* the secretin/calcitonin/PTH receptor family). Elucidation of the gene structure of the porcine calcitonin receptor (pCTR) (39) in 1994 first revealed the unique intron-exon organization of a receptor

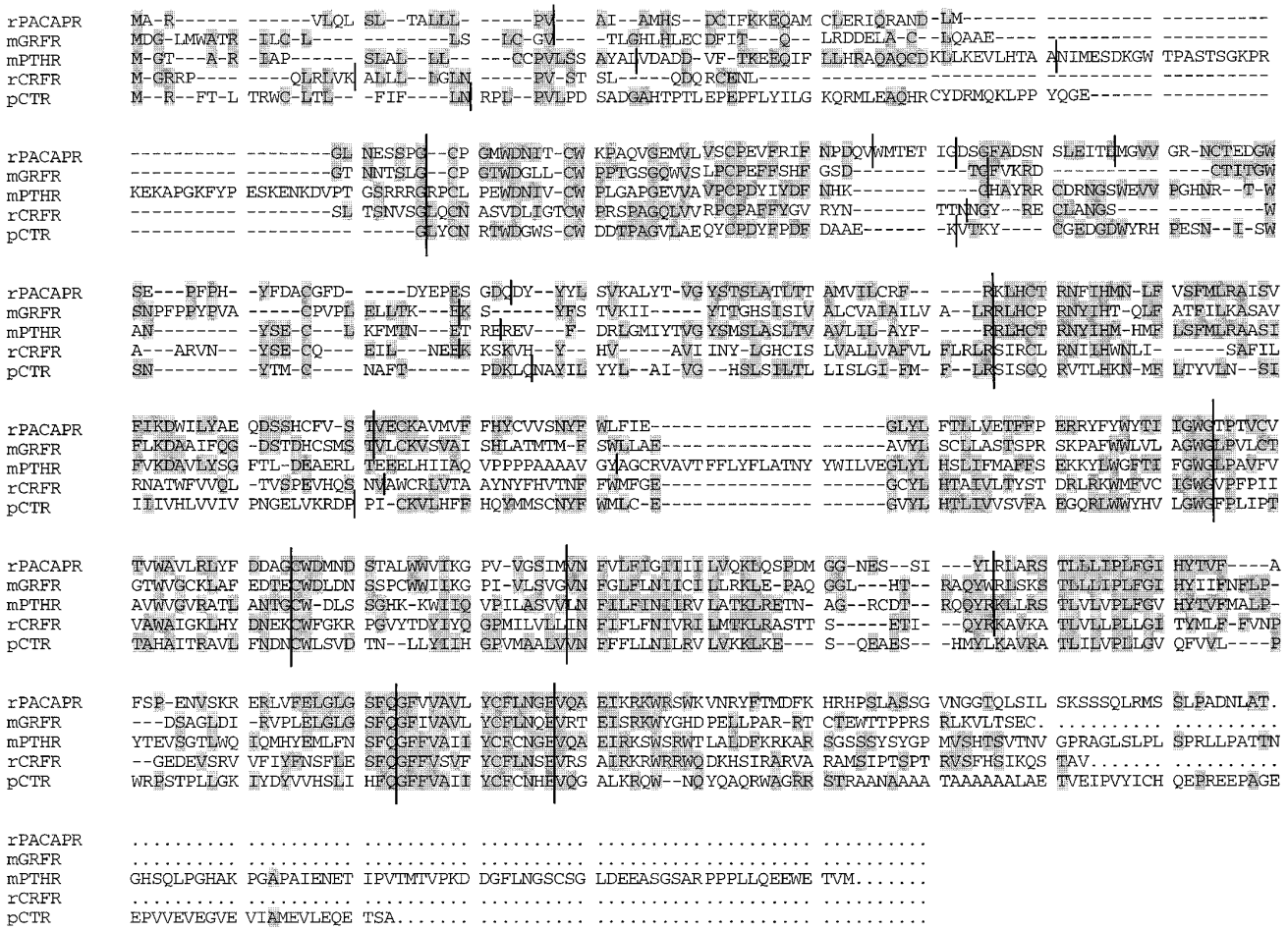

FIG. 8. **Conservation of intron-exon junction positions between the rPACAPR, mGRFR, mPTHR, rCRFR, and pCTR.** The five cDNAs were aligned to maximize amino acid identity and positions of intron-exon junction sites. The *dark vertical lines* indicate the positions of introns.

in this group of G protein-coupled receptors. By comparing the pCTR gene structure to regions of the mouse growth hormone-releasing factor receptor (mGRFR) reported to be coded for by different exons, these authors suggested a common ancestral origin of these two receptor genes. Recent studies (40, 41) elucidated the gene structures of two additional members of this receptor family, the rat corticotropin-releasing factor receptor (rCRFR) and mouse parathyroid hormone receptor (mPTHR). Fig. 8 shows the alignment of the rPACAPR exon-intron organization with that of these four receptors. As shown, there is a remarkable conservation of intron-exon junctions in these five distinct receptors from three different species. Exons encoding the transmembrane domains and intracellular regions of these receptors (*i.e.* following intron 6) have an intron-exon organization that is extremely well conserved among these receptors, whereas some variability in gene structure is apparent in exons encoding the extracellular N-terminal region. This latter finding may reflect evolution of a primordial gene to produce receptors with different ligand specificities in view of evidence indicating that the N-terminal cytoplasmic region of several members of the group III family of G protein-coupled receptors, *i.e.* the glucagon, secretin, VIP, and PTH receptors, represents the ligand binding domain of these receptors (42–44).

Genes encoding G protein-coupled receptors have been found to have either no introns (*e.g.* α_2- and β-adrenergic) (45, 46), introns only in noncoding regions (*e.g.* platelet-activating factor, bradykinin B2) (47, 48), introns only in coding regions (*e.g.* substance K, substance P) (49, 50), or introns in both coding and noncoding regions (neuropeptide Y Y1, endothelin A) (51, 52). However, apart from the luteinizing hormone receptor gene, which has 10 introns all located within the N-terminal extracellular domain, six or fewer introns are present in the coding regions of the G protein-coupled receptor genes described to date (49, 52). Moreover, none of these genes have the characteristic interruption of transmembrane domains IV, V, and VII by introns as found in the rPACAPR, pCTR, mGRFR, rCRFR, and mPTHR genes. Thus, the complex intron-exon organization of the rPACAPR, pCTR, mGRFR, rCRFR, and mPTHR genes appears to be unique to the group III family of G protein-coupled receptors.

We also provide the first evidence for alternative splicing of the PACAPR mRNA in its 5′-UTR. Using a combination of 5′-RACE and RT-PCR, we identified four rPACAPR cDNAs with unique 5′-UTR sequences. All four cDNAs are identical to each other and to the 5′ genomic sequence up to nucleotide −76, where we identified a splice acceptor sequence 5′ to this nucleotide. Three of the four cDNAs differ from the shortest cDNA (cDNA 1) by the presence of a conserved 23-bp sequence, located at different upstream positions for each cDNA. Our results show that cDNAs 2–4 are formed by alternative splicing of three exons, located approximately 8 kb (exon 1), 5 kb (exon 2), and 4.2 kb (exon 3) upstream of the translation start site, to the splice acceptor site located 5′ to nucleotide −76 (Fig. 5). One of these cDNAs (cDNA 3) is formed by splicing together of exons 1, 2, and 3 to the acceptor sequence, while the other two cDNAs are produced by skipping of one (cDNA 4) or two of these exons (cDNA 2). In contrast to the sharing of exons by cDNAs 2–4, cDNA 1 is produced by splicing to an exon containing minimally GGCAG. These findings demonstrate that

splicing occurs at several locations in the 5′-UTR of the rPACAPR mRNA to generate transcripts with unique 5′-UTR sequences.

In view of these findings, it was of great interest to compare the sequence of cDNA 1–4 and the 5′-flanking sequence of the rPACAPR gene described here to the 5′-UTRs included in reports describing the cloning of the rPACAPR cDNA. The five groups that included sequences from the 5′-UTR region of cloned rPACAPR cDNAs reported five different lengths of this sequence, *i.e.* 30, 76, 90, 335, and 396 nucleotides (26–30). Each of these sequences matches the genomic sequence described here up to nucleotide −76. The three reported sequences that extend beyond this location all diverge at this point (28–30) but match exactly the sequence of cDNA 2 reported here. cDNA 2 is the cDNA in which exon 1 is spliced directly to the 3′ acceptor sequence (Fig. 5), and our results suggest that this exon is located 8 kb upstream of the translation start site. These findings confirm that the 3′ splice acceptor site we identified here does, in fact, represent a splicing site and that splicing of the 5′ noncoding region of the rPACAPR gene to produce the 5′-UTR of cDNA 2 occurs in at least two other tissues (colliculi, olfactory bulb) (28–30). The finding that two of these sequences extend beyond the 5′ end of cDNA 2 and match each other beyond this point is consistent with our suggestion, based upon primer extension data, that cDNAs 2–4 are incomplete at their 5′ ends.

Our finding that alternative splicing of the rPACAPR mRNA can produce rPACAPR transcripts with heterogeneous 5′-UTR sequences prompted us to examine the tissue-specific nature of this phenomenon. RT-PCR analysis demonstrated differential splicing of the rPACAPR transcript to produce rPACAPR mRNAs with heterogeneous 5′-UTRs in nearly every tissue we examined. Three major products, shown by sequence analysis to represent cDNAs 2–4, were amplified from every tissue except pancreas. Thus, splicing in the 5′-UTR of the rPACAPR mRNA according to the scheme shown in Fig. 5 represents the molecular basis for formation of these heterogeneous rPACAPR transcripts. The relative amounts of these three rPACAPR transcripts, PCR-amplified using the same primer combinations, varied in different tissues, indicating differential expression of these three rPACAPR transcripts in different tissues. Further experiments will be required to determine whether the 440-bp product amplified in several tissues or the additional products amplified in kidney arise from differential splicing of exon 1 to additional exons in the 5′-UTR of the rPACAPR transcript.

At present it is unclear whether splicing of the rPACAPR mRNA in the 5′-UTR represents an important regulatory mechanism involved in tissue- or cell-specific expression, mRNA stability, or mRNA translatability as found in other genes (53–56). Alternative splicing of 5′ noncoding regions of genes for other G protein-coupled receptors, including human neuropeptide Y Y1 and A1 adenosine receptors (52, 57), seems to be related to the tissue-specific expression of their transcripts. These findings are consistent with the tissue-specific expression of differentially spliced rPACAPR transcripts observed in the present study. Presently, it is unknown whether transcripts with alternate 5′-UTR sequences are a common feature among group III G protein-coupled receptors, although there is evidence for alternative splicing of the pCTR in its coding region (39). Spengler *et al.* (30) demonstrated the existence of five rPACAPR cDNAs, representing splice variants of the receptor in the third intracellular loop region, a region shown here to be encoded by exon 12, where we identified the location of these splicing sites. No dramatic differences in the signaling activities of these coding region splice variants were observed, since all receptors stimulated adenylyl cyclase and all but one also stimulated phospholipase C. However, these findings together with the present study show that the rPACAPR gene can be alternatively spliced in both 5′ noncoding regions and coding regions.

Primer extension analysis demonstrated multiple transcription sites located from 80 to 107 bp upstream of the translation start site, with no other sites detected up to 310 bp from this site. These sites could represent heterogeneity in the transcription start site for cDNA 1 and/or the possible existence of additional transcripts, but they are insufficient in length to account for cDNAs 2–4. Indeed, these results suggest that cDNAs 2–4 represent incomplete transcripts, because no extension products corresponding to their sizes were observed. We sequenced the 5′-flanking region to 1164 bp upstream of the translation start site. This region does not contain a splicing site that can account for the generation of cDNA 1. Further studies will be required to identify the 5′-flanking regions of cDNA 1 and cDNA 4, although we do know that exon 1 is located 8 kb upstream of the translation start site.

In summary, we used a novel PCR-based strategy to amplify the entire coding region of the rPACAPR gene, determined its intron-exon organization, and provided evidence for differential splicing in the 5′-UTR of the rPACAPR transcript. Southern analysis indicated a single copy of this gene. The elucidation of the rPACAPR gene structure establishes the foundation for the use of molecular genetic approaches to further study the regulation of its transcription and splicing in the 5′ region and the role of this gene in physiology, particularly in processes such as hormone secretion and neurotransmission.

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